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TITLE: CaMKK2 Inhibition in Enhancing Bone Fracture Healing

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Fracture healing is a chief medical concern for active-duty military personnel as well as aging combat veterans, highlighting a highly critical need for novel therapies to treat this condition. A recent serendipitous discovery that genetic ablation of Ca^{2+}/calmodulin (CaM) dependent protein kinase kinase 2 (CaMKK2) has duel effects on anabolic and catabolic pathways of bone remodeling has led to the idea that its pharmacological inhibition could serve as an efficacious therapeutic strategy to promote efficient fracture healing. STO-609 is a selective, cell-permeable pharmacological inhibitor of CaMKK2 that has been successfully tested in vivo in mice. Hence the goal of this proposal is to develop STO-609-mediated inhibition of CaMKK2 as an efficacious therapeutic strategy to promote accelerated fracture healing and recovery. During the first phase of the award period, we performed a pilot study to establish the following: (1) Reliable and reproducible surgical procedures for creating a transverse femoral fracture and fixing it with an intramedullary device. (2) The treatment protocol for following the animals for the appropriate follow-up period. (3) The collection and analysis of the femur for assessing fracture healing.

15. SUBJECT TERMS
Femur fracture, CaMKK2, STO-609

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1. **Introduction:** Fracture healing is a chief medical concern for active-duty military personnel as well as aging combat veterans, highlighting a highly critical need for novel therapies to treat this condition. Genetic ablation of Ca^{2+}/calmodulin (CaM) dependent protein kinase kinase 2 (CaMKK2) has dual effects on anabolic and catabolic pathways of bone remodeling and has led to the idea that its pharmacological inhibition could serve as an efficacious therapeutic strategy to promote efficient fracture healing. STO-609 is a selective, cell-permeable pharmacological inhibitor of CaMKK2 that has been successfully tested in vivo in mice. Hence the goal of this proposal is to develop STO-609-mediated inhibition of CaMKK2 as an efficacious therapeutic strategy to promote accelerated fracture healing and recovery.

2. **Keywords:** Femoral fracture healing, CaMKK2, STO-609

3. **Accomplishments:**

A. What are the major goals of the project:

1. Specific Aim 1: Determine the optimal conditions of CaMKK2 inhibition through STO-609 that will confer the most efficacious recovery from bone fracture

2. Specific Aim 2: Effect of genetic ablation or pharmacological inhibition of CaMKK2 on post-fracture bone physiology and recovery

B. What was accomplished:

During the first phase of the award period, we performed a pilot study to establish the following:

1) Reliable and reproducible surgical procedures for creating a transverse femoral fracture and fixing it with an intramedullary device.
2) The treatment protocol for following the animals for the appropriate follow-up period.
3) The collection and analysis of the femur for assessing fracture healing.

We used 12 eight-week old male C56BL6 mice that were purchased from a vendor for this pilot study. After being anesthetized with a cocktail of ketamine and xylazine and throughout the procedure, mice were kept on a warming pad. Eyes were lubricated with artificial tears ointment. The area around the left anterior distal femur was disinfected through the application of 70% isopropanol and the hair was shaved. The operative site was shaved and disinfected with alternating scrubs of betadine and alcohol. Sterile instruments were used to make a small incision (approximately 2 mm). A 25 gauge needle (0.455 mm, intramedullary pin) was inserted retrograde into the intercondylar notch of the left femurs of anesthetized mice through a trephine technique and advanced through the intramedullary canal to the trochanteric region of the femur. Following insertion of the needle in the femur, a digital x-ray was obtained to confirm accurate needle placement. Next, a closed, unilateral mid-shaft fracture was generated using a modification of a method first described for use in rats by Bonnarens and Einhorn, 1986 (*11, 18*) using a custom fracture three-point-bending device (Figures 1-2).

Physical examination was performed and a second digital radiograph was obtained immediately after the fracture procedure to confirm the location and quality of fractures (Figure 3). The imaging was performed using the micro-CT in the Orthopaedic Bioengineering Lab (Voor lab) in digital radiograph mode while the animals remain anesthetized immediately after surgery. Mice with comminuted or non-mid diaphysis fractures were excluded from the study and euthanized. When fractures were acceptable, skin incisions were closed with sutures. The non-fractured contralateral femur from the same mouse served as controls in all the experiments. The mice were treated with antibiotics during the initial period following surgery and provided with analgesics, daily for five days and as needed, thereafter.

The mice were divided into two groups of 5. From the day following surgery, one cohort received tri-weekly injections of sterile saline (200 µl/mouse) for 6 weeks, while the other cohort received tri-weekly injections of STO-609 (10 µmoles/kg body weight; 200 µl/mouse) for 6 weeks. At the end of the 6-week period, mice were euthanized and fractured and contralateral control femurs were isolated and dissected clean of all soft tissues. The bones were then wrapped in saline soaked gauze or placed in 10 % formalin. Later the femurs were scanned using micro-CT at 7 micron voxel size for volumetric analysis of the healing fracture callus. We are currently performing the 3D volumetric analyses to calculate total volume of callus, along with BV/TV, maximum area and polar moment of inertia. After completion of these analyses, we will perform the dose response curve analyses of STO-609 to determine the optimal conditions of CaMKK2 inhibition that will confer the most efficacious recovery from bone fracture.

Figure 1: Device that utilizes a 3-point bending strategy to perform closed fractures in mice was assembled.

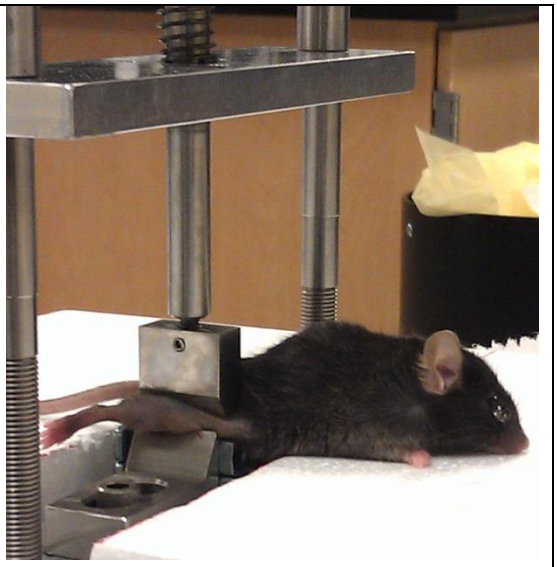

Figure 2: Generation of femur fracture in mice using the device

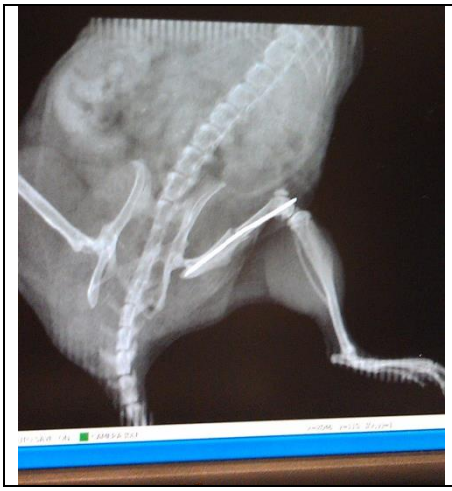

Figure 3: Succesful generation of a unilateral mid-shaft fracture on the pinned femur using the device.

C. What opportunities for training and professional development has the project provided:

Nothing to report.

D. How were the results disseminated to communities of interest?

Nothing to report.

E. Plan for the next period:

We will perform experiments to determine the optimal conditions of CaMKK2 inhibition that will confer the most efficacious recovery from bone fracture

4. Impact

Nothing significant to report for this project period.

5. Changes/Problems

Nothing to report.

6. Products

Nothing to report.

7. Participants and other collaborative institutions

PI: Uma Sankar, Ph.D.

Associate Professor, Pharmacology and Toxicology, University of Louisville, KY.

Nearest person months worked: 2

Contribution to the project: Designed the study and interpreted the results. Wrote the report.

Co-investigator: Michael J. Voor, Ph.D.

Associate Professor, Orthopaedic Surgery, University of Louisville, KY.

Nearest person months worked: 2

Contribution to the project: Designed the study and interpreted the results.

A. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

B. What other organizations were involved as partners?
Nothing to report.

8. Appendices

None.